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Resistance to bacterial wilt pathogen of maize and genetic host-parasite interactions

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RESISTANCE TO BACTERIAL WILT PATHOGEN OF MAIZE AND GENETIC HOST-PARASITE INTERACTIONS

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INTRODUCTION

Bacterial variation has been studied mainly *in vitro*, few investigations having been made of the effect of host passage on the pathogen. The common belief is that virulence is maintained or enhanced by host passage. Outstanding as a critical analysis of this problem is the work of Wellhausen (26) who determined the effect of repeated passage of *Bacterium stewartii* (E.F.Sm.) Stevens* through resistant and susceptible lines of maize and through certain other grasses. Successive passage through susceptible maize decreased virulence, while successive passage through resistant maize increased virulence. Passage through teosinte had an effect comparable to passage through susceptible maize. Passage through 14 other grasses increased the virulence of *B. stewartii* for the particular grass, but decreased it for maize.

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* This species was first studied and named by E. F. Smith. He named it *Pseudomonas stewarti* (17), later (18) changing the genus to *Bacterium*. When McCulloch (13) demonstrated its non-motility, she named it *Aplanobacter* according to Smith's classification. Stevens (20) was the first to publish the name as *Bacterium stewarti* (E.F.Sm.) E.F.Sm. drawing attention to its non-motility. At present it is called *Phytomonas stewarti* (E.F.Sm.) S.A.B. Elliott (3) states that by priority the name *Phytomonas* belongs to a protozoan, native to the latex of plants. Since no one name is generally accepted as satisfactory, the conservative usage is employed here.

This investigation has been devised to check and extend Wellhausen's observations on the effect of the host upon bacterial virulence, to determine how virulence changes, and to use bacterial virulence as a tool to determine the nature of host resistance.

REVIEW OF LITERATURE

E. F. Smith (18), the first to study B. stewartii, considered it to be very stable in culture. In 1918 McCulloch (13) reported two distinct types of colonies, one with a smooth surface, the second with a central depression or crater, both remaining stable for at least 2 years. Other workers, (8, 9, 29) report that atypical strains of B. stewartii may be isolated from infected soil or plants. Recently McNew (14) isolated single colonies from dilution plates of virulent cultures of B. stewartii and found stock cultures to be composed of many variants. Later, working with 10 strains of B. stewartii differing in virulence McNew (15) found the strains also differed in their ability to utilize nitrogen from inorganic sources; to reduce nitrates to nitrites; and to produce a curd in litmus milk. The ability to use inorganic nitrogen was correlated positively with virulence. Ivanoff et al. (10) from their studies of 22 cultures of B. stewartii report that this species included several types of organisms which they separated into 3 main strain types on morphological, physiological and colony characteristics. A positive correlation between amount of gum and pathogenicity was indicated. A similar correlation between consistency of growth on agar and virulence also was observed by Wellhausen (25, 26).

Allen and Baldwin (1) determined the effect of repeated plant passage of effective and ineffective strains of Rhizobium trifolii, Rh. leguminosarum, and Rh. japonicum. They report that effective strains may become ineffective, or ineffective strains become effective by repeated plant passage. In some experiments the change in efficiency came in one passage, in other experiments it was gradual.

Hendrickson et al. (7) determined the effect of passage of the crown gall bacterium through the tomato upon the physiological characteristics and virulence of the organism. Variation in the physiological behavior of the crown gall organism was obtained by plant passage, but no effect upon virulence was noted.

Reports on experimental epidemiology give indications that host passage modifies bacterial virulence. Webster and Clow (23) found that strains of Pneumococcus vary widely in virulence as judged by internasal instillations, and these differences are often not parallel with the degree of virulence in interperitoneal inoculation. Serial passage from nose to nose consistently failed to raise the internasal virulence of initially low virulent strains; serial internasal passage of strains with high initial virulence reduced internasal virulence almost to zero without inducing any change in the interperitoneal virulence. Earlier, Webster (22) had observed

that virulent strains of Pasteurella lepiseptica may be replaced in the nasal passages of rabbit by relatively avirulent strains.

With mouse Pasteurella, Greenwood et al. (5) showed that avirulent strains may arise by passage through immunized animals, whereas strains obtained from non-immunized mice were as virulent as the original strain. In a case of Bacillus aertrycke, a virulent epidemic arose by emergence of a virulent variant from a relatively avirulent strain.

The criticism of most investigations on host passage is that host resistance has not been kept a constant, hence the cause of any bacterial variation is difficult to interpret.

EXPERIMENTAL

Materials

The host material for these virulence studies was limited largely to 4 lines of maize inbred for at least 10 generations. A yellow-dent line, OSF, was the resistant host, while a white-flint line, WF, and two yellow-sweet lines, GB134 and GB797, were used as highly susceptible hosts. GB134 was used in passage work, while GB797 was used for all virulence tests. Other inbred lines were used in some experiments, and their resistance reaction will be given later.

Resistant line, OSF, when inoculated with B. stewartii showed characteristic lesions only on those leaves actually expanded at the time of inoculation, subsequent leaves being free of lesions. Stunting of the plant was slight. Susceptible lines, GB134 and WF, die soon after inoculation, usually within two weeks. With these lines the initial symptoms are the characteristic wilt lesions, with the bacteria soon becoming systemic throughout the vascular system resulting in a wilted condition soon followed by drying and death. GB797 reacts like GB134 or WF but is slightly more resistant and dies somewhat slower. Wellhausen (27) determined the genetic

basis of resistance between these lines as being primarily influenced by 3 supplementary dominant factors.

Several cultures of B. stewartii were tested for virulence, and from this group, six cultures that represented a range of virulence from high to low were selected for further use. As a check on their purity all cultures were plated, observed, and a composite of 15 typical colonies used to make up a stock culture. After this purification the stocks were continued on nutrient dextrose agar. Cultures were transferred once every 4 weeks, allowed to grow at room temperature for 24 hours, then stored in the refrigerator at about 5° C. Strain characteristics are summarized in table 1. On nutrient dextrose agar the more pathogenic strains form larger, more viscid or mucoid colonies than do the less pathogenic strains.

The writer secured these cultures through the courtesy of Drs. E. J. Wellhausen, S. S. Ivanoff, C. B. Elliott, and J. H. Muncie.

TABLE 1. Growth characters on nutrient dextrose agar of bacterial stocks used for host passages.

Culture Used	Virulence Index*	Characteristics of 48-Hour Agar Plate Colonies				Generation Time in Hours***
		Diameter in Microns	Consistency	Surface	Color	
S15	64	94 ± 6**	Dry, Granular	Rough	Dark orange-yellow	2.3
D3	55	139 ± 26	Dry, Granular	Rough	Lemon yellow	2.9
A87	52	304 ± 87	Butyrous	Smooth	Lemon yellow	2.0
FB32	40	220 ± 44	Butyrous	Smooth	Creamy yellow	2.2
A14	23	550 ± 76	Mucoid	Smooth	Creamy yellow	1.8
023	28	302 ± 73	Butyrous-viscid	Smooth	Creamy yellow	3.2

* Virulence index = $\frac{\text{Green wt. of test plants}}{\text{Green wt. of check plants}}$ x 100

** Standard deviation calculated on 300 colony measurements

*** See pages 52-53 for methods.

Methods of Procedure

Bacteria for inoculum were grown on agar slants for 24 hours, washed off into 5 c.c. of nutrient broth and without further incubation inoculated into the test plants. Plants for host passage were grown in greenhouse flats, five or six rows to a flat. Host plants were inoculated by injecting a small quantity of inoculum into the first nodal region of the plant with a hypodermic syringe. Since the amount of inoculum for each plant was uncontrollable, standardization of the inoculum was considered unnecessary. The actual amount of inoculum consists of the liquid remaining in the needle hole after the syringe needle is withdrawn. Percentage of infection is greater than 99 per cent. Reisolations were made 14-16 days after inoculation, using 8-10 plants from each row. A short section of the third or fourth leaf was taken from each plant, surface sterilized by dipping in 95% alcohol for 4-5 seconds, passed through a flame and dropped into sterile water. These sections were then macerated in a small quantity of sterile water, the mass mixed thoroughly, about 1 c.c. of this liquid put into sterile broth, shaken repeatedly, and dilution plates poured. To observe colony type changes, surface colonies were obtained by means of a poured-plate smear technique.

After reisolation the new passage culture was made up of a large number of colonies (from 50-100) picked at random and pooled into one culture. Such a large number of colonies was used to insure a more representative sample of the population than one or a few colonies would give. After 24 hour's growth at room temperatures, the passage culture was either used as inoculum for the next passage, or in case the plants for that passage were not ready the culture was stored in a refrigerator at about 5° C.

In passage experiments, virulence was determined after 2, 4, 6 and 8 passages on a susceptible inbred, GB797. Test plants were grown in greenhouse benches. Not less than 15 plants were inoculated with each culture. Each test culture was replicated 3 times and compared in virulence with the virulence of the initial stock culture. After the 8th passage all cultures were given comparative tests against the stock culture and against each passage stock. Significance was tested on the observations using an analysis of variance design (19).

Green weights of each block of plants were taken 14 days after inoculation. A virulence index was calculated as

$$\frac{\text{Green weight of test plants}}{\text{Green weight of check plants}} \times 100.$$

Green weight means the weight of the top above the first node. Another virulence index has been used and described by McNew (14) and Wellhausen (26).

The procedure in designating passage cultures has been to list the stock culture, the maize line through which passed, and the number of passages. Thus S15-GB-8 means the culture obtained by passage of stock culture S15 through maize line GB134 for 8 successive passages.

Experimental Results

Effect of successive host passage on the virulence of six stock cultures.

Subcultures of six stock cultures were passed successively through one resistant and two susceptible maize lines. Tests for virulence were made after 2, 4, 6 and 8 passages. A virulence comparison with the original strain was made at each test, and after 8 passages all passage cultures were compared with each other and with the stock strain. Virulence of the stock culture and of the passage strains is given in table 2. There is a pronounced trend for decrease of virulence of the subcultures by passage through the susceptible lines GB134 and WF, and for increase by passage through the resistant line OSF. To such a generalization there is only one exception - that of the FB32-WF passage.

The S15 and 023 passage series are shown in plates 1 and 2 respectively, where the change in virulence during passage is a progressive shift beginning immediately on passage. In the S15 passage, the ultimate reduction in virulence is brought

TABLE 2. Virulence indices of stock and passage cultures during 8 successive passages through susceptible and resistant hosts.

Host Line	Number of Host Passages	Virulence Indices of Strains S15	D3	A87	FB32	023	A14
GB134 (susc.)	0	64	55	52	40	28	23*
	2	73	68	63	52	48	46
	4	81	84	69	50	50	55
	6	79	88	83	67	69	64
	8	82	90	83	73	81	71
Difference		+18	+35	+31	+33	+53	+48
Differences of less than 20 are not considered significant							
WF (susc.)	0	64	55	52	40	28	23
	2	61	59	57	36	30	38
	4	78	54	63	29	53	46
	6	75	47	59	32	65	64
	8	81	67	67	35	72	72
Difference		+17	+12	+15	- 5	+44	+49
Differences of less than 23 are not considered significant							
OSF (Res.)	0	64	55	52	40	28	23
	2	55	57	28	46	22	29
	4	42	48	32	37	32	28
	6	40	39	30	28	30	29
	8	44	37	33	35	26	27
Difference		-20	-18	-19	- 5	- 2	+ 4
Differences of less than 18 are not considered significant							

* This is a second series. In the first series the passage culture became completely avirulent apparently due to the culturing of a contaminant.

about within the first 4 passages through the susceptible line. Through the resistant line, increase in virulence was most rapid in the early passages but apparently was still rising when the experiment was stopped.

Change in virulence during passage was associated with changes in colony growth on nutrient dextrose agar plates. Loss in virulence was always associated with a raised firm type of colony. Of the four initially smooth cultures passed through the susceptible host - A87, FB32, 023 and A14 - in only A14 was the change from smooth to rough phase associated with the decrease in virulence; in the others the colonies remained in the smooth phase, becoming drier, firmer and more intensely colored. The initially rough S15 and D3 cultures decreased in virulence during susceptible host passage remaining in the rough phase without apparent colony type changes.

Associated with an increase in virulence by passage through the resistant host, agar colonies became more spreading, watery and viscid. The change from rough to smooth was observed in passage cultures of both the initially rough stocks, and the increase in the proportion of smooth type was associated with the increase in culture virulence. The highly virulent A14 and 023 cultures remained constant on resistant host passage; A87 and FB32 growth became more watery and spreading as virulence increased.

Change of virulence in passage stocks by reversing hosts

To determine whether the virulence of a passage culture could be again changed by reversing the host through which it

is passed, virulent passage strains which had been passed through resistant OSF for 8 times were successively passed through susceptible GB134; and the sister avirulent passage cultures obtained after 8 passages through GB134 were passed through OSF. Six successive reverse passages through the new host were made, and virulence determined after 2, 4 and 6 passages. (Table 3).

During these reverse passages, changes in colony type and virulence were not different from those described in an earlier paragraph. Virulence of passage stocks changes as readily and rapidly as stock cultures that had had no host passage.

TABLE 3. Virulence change of passage cultures after reversing the host through which bacterial stocks were passed.

Host Line	Number of Host Passages	Virulence Indices of Strains			
		S15-GB-8	A87-GB-8	023-GB-8	A14-WF-8
OSF (Res.)	0	82	83	81	72
	R2	76	64	43	60
	R4	59	44	36	53
	R6	49	34	30	41
Difference		+33	+49	+51	+31
Differences of less than 16 are not considered significant					
		S15-OSF-8	A87-OSF-8	023-OSF-8	A14-OSF-8
GB134 (Susc.)	0	44	33	26	27
	R2	57	48	50	35
	R4	64	58	63	56
	R6	72	65	68	65
Difference		-28	-32	-32	-38
Differences of less than 13 are not considered significant					

Virulence variation within a stock culture, and the effect of successive host passage on virulence variability

As a further test of what changes take place within a bacterial population, a stock culture of intermediate virulence was selected to test the virulence-variability initially present in the culture after 8 passages through susceptible and resistant hosts. Culture A88, selected for this purpose, had been held on agar slants for 3 years with no plating or host passage. One hundred single-cell isolates were obtained by plating following the method described by McNew (16). Each colony was plated successively for 3 times, then put on agar slants and held as a stock culture. Virulence tests were made in triplicate within a week after single-cell cultures were obtained.

The 100 single-cell cultures from stock A88 varied widely in virulence with most of the colonies tending to be of about the same general virulence as that of the parent stock (figure 1, line A). A wide range of virulence was found in isolates from this culture. Mean virulence of the 100 isolates was 53 with a standard deviation of ± 21. With respect to virulence this stock culture is a population composed of many variants. When such a culture is inoculated into maize the host is subjected to invasion by a great number of bacterial variants. If certain variants are better adapted to the micro-environment of the host, selective growth would occur, changing the composition of the population.

Such is actually found to be the case, for after 8 passages through the susceptible line the mean virulence of the passage culture had changed from 52 to 83. The mean virulence of 100 single-cell isolates from this passage culture was 69 with a standard deviation of ± 16 (figure 1, line B). Passage through the susceptible host effected a definite shift in the bacterial population toward the avirulent type of organism. This is consistent with previous results.

With passage through the resistant line the mean virulence changed after 8 passages from 53 to 33 (figure 1, line C). The mean virulence of 100 single-cell isolates from this passage culture was 36 with a standard deviation of ± 15, again with a reduction in variance.

The change in virulence of the passage cultures is due to a shift in the ratio of virulent to less virulent forms initially present. This is contrary to concepts held by certain bacteriological pathologists. For example, Wilson (28) states, "When a given strain comprises organisms showing discontinuous variation in virulence, the virulence of the whole culture is similar to that of the most virulent variants."

To test this hypothesis a simple experiment was made with virulent and avirulent strains. Selected single-cell strains of A14 (virulent) and S15 (avirulent) were mixed by dilution to give the range from zero to 100% of each type without regard to actual proportionate number of virulent and avirulent bacteria

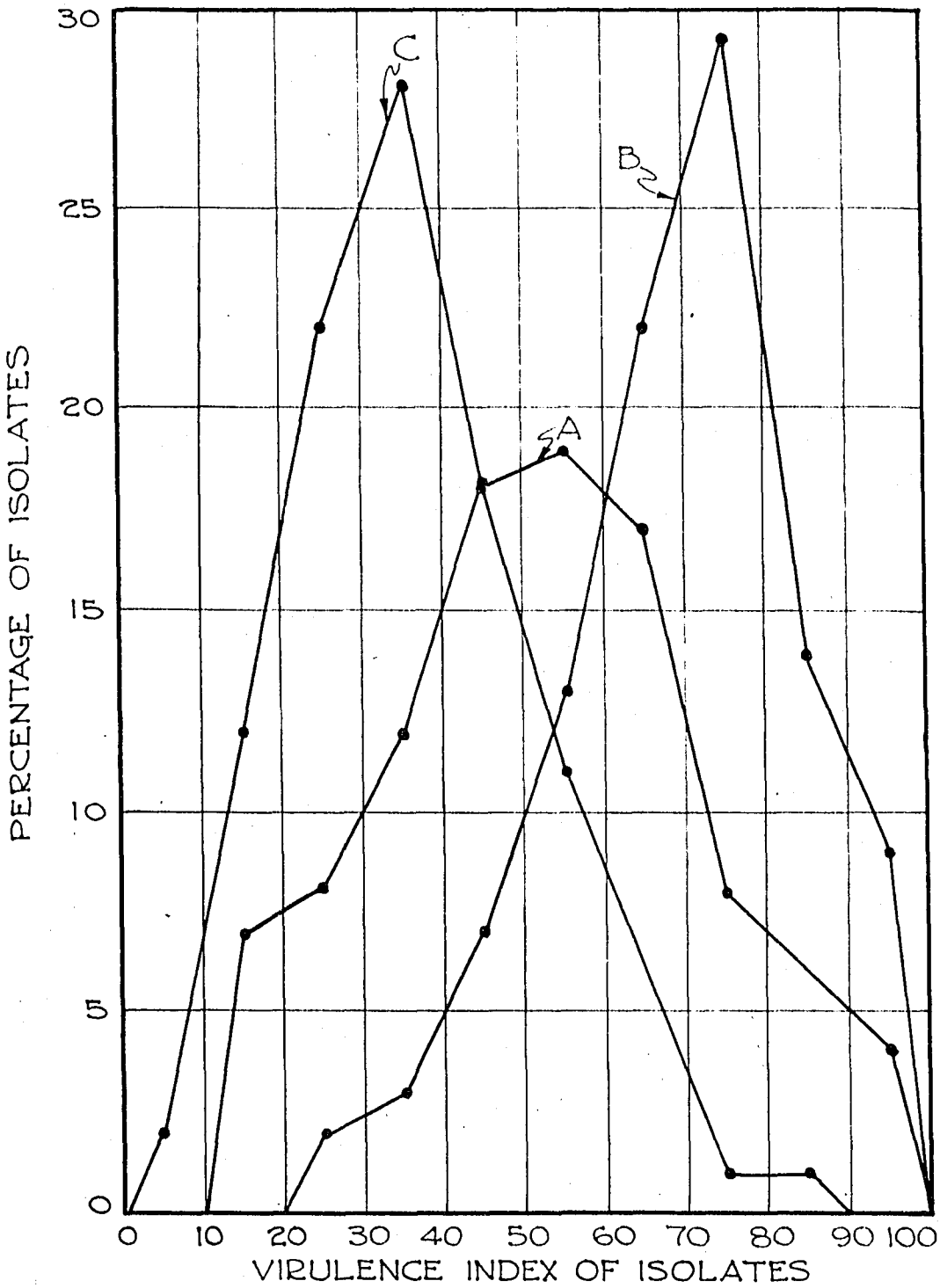

Figure 1. **Frequency distribution of pathogenicity of single-cell isolates of B. stewartii from a culture before and after host passage.**

present. As shown in figure 2 the virulence of the culture bears a direct relationship to the dilution of virulent to avirulent bacteria. Therefore the proportion of virulent and avirulent types in the inoculum determines the virulence of that culture and not the virulence of the most virulent variant. There is no indication of an "all or none" reaction at any dilution in this organism.

Host passage of single-cell isolates

Bacterial stocks derived from single cells should be uniform for all characters if it be assumed that bacteria multiply only by simple fission. In such a culture all individuals would be of the same virulence, and selection should be ineffective unless variations arose. A critical test of whether virulence change is due entirely to selection of existing variation or whether adaptive variation can arise by some other means is furnished by host passage of single-cell cultures. Such a test was made by repeatedly passing virulent or avirulent single-cell cultures through both resistant and susceptible maize lines and determining the effect on virulence and colony type.

Single-cell cultures were obtained using the method described by McNew (16). Three successive platings of each colony were made. Accepting his probability of a colony of B. stewartii developing from a single cell (99.0 ± 0.35 to 1)

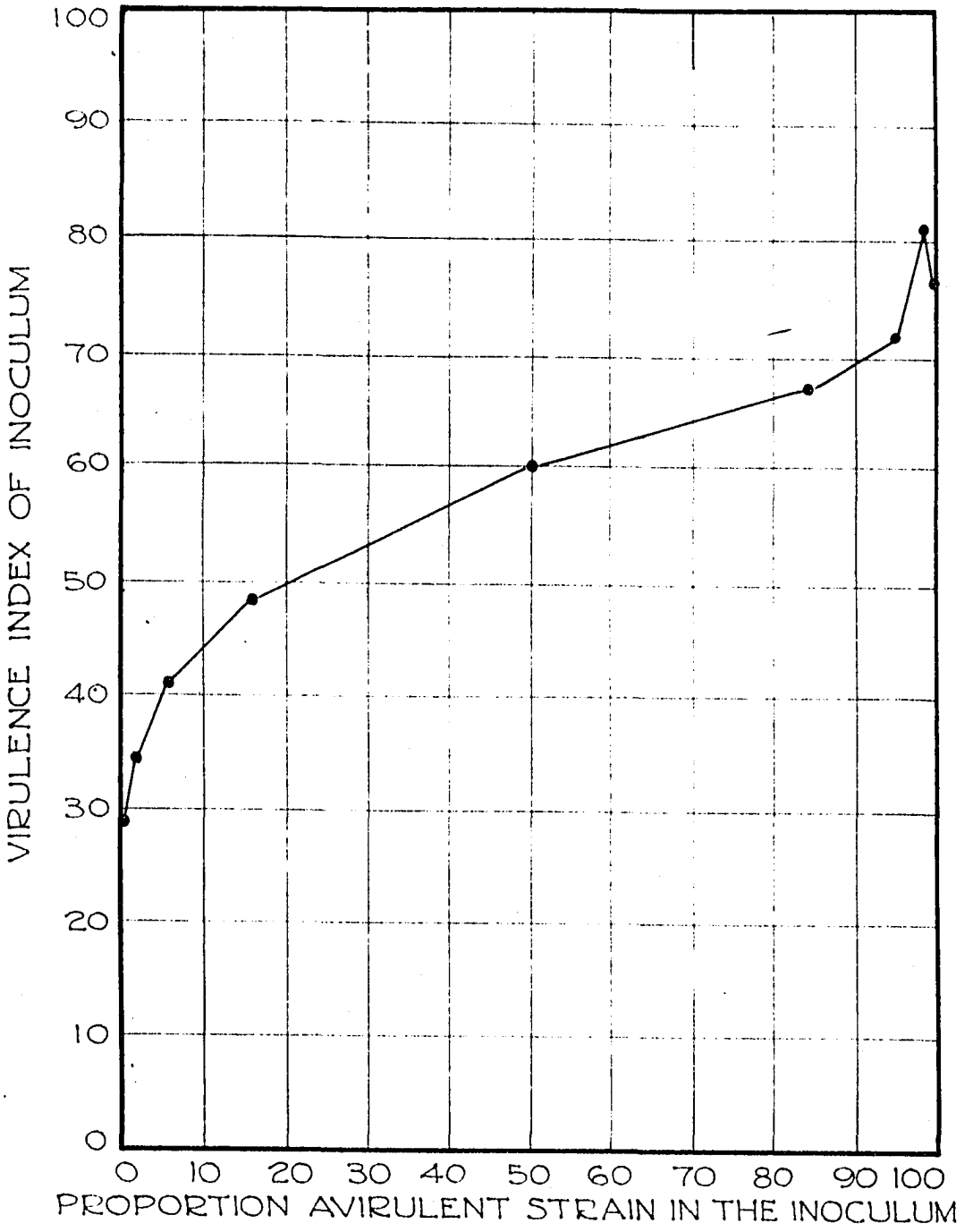

Figure 2. **Change in virulence of inoculum composed of various proportions of virulent and avirulent bacteria.**

the chance of picking a colony not derived from a single cell at least once during the three platings is very low. Single-cell strains were obtained from the virulent A14 stock. Each single-cell culture was passed through a resistant and a susceptible host using the technique of host passage described earlier. Passage was made for 6 successive times, virulence tests being made after 2, 4 and 6 passages (Table 4).

Not all cultures were passed through the resistant host since previous work had indicated the lack of any noticeable change due to passage. Perhaps the most striking thing observed with these single-cell culture passages is the consistency with which all cultures passed through the susceptible line decreased appreciably in degree of virulence, while those passed through the resistant line retained their virulence without appreciable change. Notes on the particular passage in which atypical colony types were first observed are interesting and believed to be significant in showing that the atypical colony type was not present at the beginning of the passage, and its origin, by whatever source, appears to be a process occurring rapidly in some cultures, more slowly in others. In only one passage culture were atypical (rough-mucoid) colony types observed immediately after the first passage, and in only one culture were atypical colonies not observed during the 6 passages.

Single-cell stocks of A14 during passage shift in virulence and colony type in a manner comparable to the change

TABLE 4. Virulence indices and observed variation of single-cell cultures f successive host passages.

Stock Number	Initial Virulence Index	Virulence Indices After Host Passage							
		Susceptible Host				Resistant Host			
		Passage Number				Passage Number			
		2	4	6	Difference*	2	4	6	Differenc
Stock Culture A14	23	44	56	67	+44	25	21	20	-3
Single-Cell Cultures									
502	20	22	49	58	+38				
504	17	19	26	37	+20	21	20	23	+6
507	27	40	69	64	+37				
508	16	31	34	38	+22	25	24	20	+4
510	16	20	23	37	+21				
511	18	27	35	46	+28				
512	22	46	43	51	+29	23	27	21	-1
513	19	17	25	32	+13				
516	15	42	59	76	+51	21	18	26	+11
517	30	33	43	47	+17				
519	29	32	37	34	+ 5				
520	18	21	35	50	+32	23	25	20	+2
522	17	26	60	64	+47				

* Differences of less than 15 are not considered significant

)served variation of single-cell cultures from A14 stock after six
s.

rulence Indices After Host Passage					Colony Variants	
)le Host	Resistant Host					
	Passage Number					
Difference*	2	4	6	Difference*	Passage First Observed	Virulence Indices
+44	25	21	20	-3	2	71
+38					3	87
+20	21	20	23	+6	3	57
+37					1	55
+22	25	24	20	+4	3	52
+21					5	50
+28					5	76
+29	23	27	21	-1	4	58
+13					3	46
+51	21	18	26	+11	2	65
+17					5	55
+ 5					none	--
+32	23	25	20	+2	4	61
+47					2	70

not considered significant

of the original A14 stock culture. This observation somewhat parallels Hadley's (6) general statement on bacterial dissociation. He states that the more recent work on bacterial dissociation, starting with single-cell cultures is in no way different from earlier results which involved only colony isolations or in some cases apparently only mass inoculations.

Differential host selection of virulent and avirulent bacteria

Since certain virulent strains of B. stewartii are characterized by one type of colony and avirulent strains by an entirely distinct type of colony, in a mixture of these two, a morphological character may be used to identify the physiological potentiality (virulence) of a given bacterium. In the previous experiments the fact that host constitution plays a definite part in the degree of virulence ultimately attained in the bacterial population has been demonstrated. An experiment was designed to furnish quantitative data on the rate at which the change in a bacterial population takes place during host passage when the initial variance of the population is definitely known and can be followed in time.

A virulent strain of B. stewartii, characterized by large, smooth, spreading, mucoid type colonies (plate III, b) was mixed with an avirulent strain characterized by smaller, slightly rough, raised non-mucoid colonies (plate III, a). The initial proportion of virulent to avirulent colonies was determined on agar plates. Such a mixture was then inoculated into resistant

(OSF) and susceptible (GB134) lines of maize. The subsequent proportions of virulent to avirulent types were then ascertained by isolating at suitable intervals of time from the host. For each isolation, lesions from 10 or more plants were macerated together in a small quantity of sterile water, about 1 c.c. of this liquid put into broth, shaken repeatedly over a period of about one-half hour, then dilution plates poured from this broth suspension. Surface-smeared plates were used, and only plates which had from 150 to 250 colonies were counted. All colonies were observed and counts made with a binocular, dissecting microscope at a magnification of 16X. Not less than 400 colonies for any isolation and usually 750-1000 colonies were counted. Thirteen such experiments were completed, an effort being made to get a wide range in the proportion of virulent to avirulent organisms, and to spread the experiments over several months, minimizing environmental variations. In terms of percentage of the avirulent type of bacteria in the initial inoculum, the range actually obtained varied from 2 to 85 per cent. Passage time varied from 14 to 21 days. All experiments were paired, that is, any initial mixed inoculum was always passed through both the resistant and the susceptible lines. In six experiments isolations were made 4 or 5 days after inoculation; in the others isolations were made only at the end of the experiment. The data for these experiments are presented

in tables 5 and 6 respectively.

Strains of A14 and S15 were used for most of the mixed culture work. These were particularly favorable because they represented extremes of virulence, were very stable in culture and were distinct in colony morphology (see plate 3).

In table 5 are included the experiments in which isolations were made repeatedly at chosen intervals of time after inoculation. There appears to be a slight tendency for the proportion of avirulent type organisms to drop immediately after inoculation. This is during the period when the first lesions are forming, and the bacteria are becoming established. It may be that this measures in some degree certain factors which influence the growth of the bacteria but whose influence disappears after the bacteria become fully established in the host. Such a possibility would still not change the nature of the conclusions drawn from these data since the net change is large and consistent. No curvilinear trend was detected in these data. Changes in the proportion of virulent and avirulent types of bacteria proceeded in orderly linear fashion from the time of inoculation to the death of the host.

There is a correlation of $r = 0.95 \pm 0.02$ between the ratio of virulent and avirulent bacteria in the inoculating suspension and that obtained by isolating from the first definite lesion on the leaf, showing that bacteria which

TABLE 5. Progressive change in proportion of avirulent type colonies during passage of mixed culture through susceptible and resistant hosts and rate (regression) of change.

Date 1938	Initial Proportion of Avirulent Type Colonies	Susceptible Host-GB134 Proportion -- Days After Inoculation				Regression	Resistant Host-OSF Proportion -- Days After Inoculation				Regression
		5	9	13	18		5	9	13	18	
Mar.11	15	20	23	-	33	+1.05	15	14	14	11	-0.24
Mar.11	39	35	40	-	52	+0.76	34	36	29	25	-0.82
Mar.11	76	70	68	74	80	+0.23	67	57	55	49	-1.00
		Days After Inoculation					Days After Inoculation				
		6	10	15	21		6	10	15	21	
May 22	2	1	8	18	21	+0.95	4	2	1	1	-0.05
May 22	28	27	33	40	43	+0.75	24	20	14	11	-0.75
May 22	76	74	70	79	80	+0.20	71	66	44	32	-2.20

TABLE 6. Change in proportion of avirulent type colonies after passage of mixed cultures through the resistant and susceptible host, with average daily rate of change.

Date 1938	Duration of Passage in Days	Initial Proportion of Avirulent	Susceptible Host-GB134		Resistant Host-OSF	
			Proportion of Avirulent	Average Daily Rate of Change	Proportion of Avirulent	Average Daily Rate of Change
Mar. 15	14	35	60	+1.96	28	-0.55
Mar. 15	14	15	32	+1.33	12	-0.22
Apr. 1	19	59	72	+0.72	46	-0.72
May 8	14	20	37	+1.30	14	-0.49
May 8	14	72	79	+0.52	62	-0.73
May 22	20	35	51	+1.45	21	-0.74
Aug. 5	15	85	92	+0.50	63	-1.49

actually grow and produce a diseased condition are reliably estimated by plating directly from the inoculating medium.

Upon passage of the mixed cultures through the susceptible host there is a differential selection for the avirulent type of bacteria. The rate of change is slow in a population containing a high proportion of avirulent organisms - rapid in a population containing a low proportion of avirulent organisms.

On passage through the resistant host there is a differential selection for the virulent type of bacteria, instead of the avirulent as in the experiment above. When the initial proportion of virulent to avirulent bacteria was 50:50, the proportion observed after a 15-day passage averaged 63:37. The inoculum containing a high proportion of virulent organisms changes at a slow rate toward the limit of 100 per cent virulent organisms. The inoculum containing a low proportion of virulent organisms changes rapidly toward one of higher proportions.

When the rate of change in the avirulent type of bacteria was determined on the original data all rates were positive after passage through the susceptible, negative after passage through the resistant line of maize. The slope of these rates is linear and, as determined by regression, is equal to -1.1 in the susceptible host and -1.7 in the resistant host (11). An inoculum with a high proportion of avirulent organisms upon passage through the susceptible line changed at a slow rate of

+0.4, but the same inoculum on passage through the resistant host changed rapidly toward a higher proportion of virulent types at a rate of -1.9. The converse would be true for an inoculum with a low proportion of avirulent bacteria.

The conclusion that intensity and direction of selection of the pathogen in the host are dependent upon host resistance has been verified by using 14 other inbred lines of maize as host. The same technique and bacterial stocks were used in these experiments as in the previously described ones, but new host-lines with different degrees of resistance were used. Host resistance as rated by Wellhausen (24) ranges from 1 to 5, with 1 representing a degree of resistance in which there is no noticeable stunting of the inoculated plants and 5 representing extreme susceptibility, plants dying in 10-20 days after inoculation with a virulent strain of bacteria. Ratings 2, 3 and 4 are intermediate stages. Data on change in the proportion of avirulent bacteria after passage, together with the rated resistance of the line are given in table 7.

It is again apparent that direction and rate of change in the proportion of avirulent bacteria during passage is dependent upon host resistance. There is a high correlation between host resistance, rate and direction of change in the bacterial population. The direction of change in all lines of intermediate to low resistance (susceptible) -- rated 3, 4 and 5 --

is toward a higher proportion of avirulent organisms. In resistant lines, rating 1 or 2, the direction is toward a lower proportion of avirulent organisms. One exception to this is noted - that of BM, a resistant, early sweet corn inbred from the Black Mexican variety. This line gave a selective reaction similar to that of a highly susceptible line. An explanation is not available at present.

Another test of host selection is to vary the virulence of the bacteria in a mixed culture used to judge selection rate and direction. Using bacterial virulence as the variable influencing selection's rate and intensity, a group of 7 bacterial stocks that gave a virulence index range from 20 to 86 were each mixed with a common avirulent stock (S15), passed through the same host, and the direction and rate of change in the bacterial mixture determined. GB134 and OSF were the maize lines through which these mixtures were passed.

All bacterial stocks were chosen both for a colony type and virulence that was very distinct from S15 stock. Of the 8 stocks used, S15 and A14 have already been described. RM is a rough mucoid variant observed to arise from A14 during passage through the susceptible line. All other stocks were selections from the A88 experiment, the summarized data of which were presented in an earlier section. The technique of mixed culture selection is the same as described in the beginning

TABLE 7. Change in proportion of avirulent type colonies after passage through various maize lines of known resistance to Bacterium stewartii, with average daily rate of change.

Maize Line or Cross	Resistance of Line*	Test 1. 19 Day Passage: Proportion of Avirulent Type: Initial	Test 1. 19 Day Passage: Proportion of Avirulent Type: Final	Test 1. 19 Day Passage: Average Daily Rate of Change	Test 2. 21 Day Passage: Proportion of Avirulent Type: Initial	Test 2. 21 Day Passage: Proportion of Avirulent Type: Final	Test 2. 21 Day Passage: Average Daily Rate of Change
GB134	5	59	72	+0.7	35	51	+0.8
887	5	59	76	+0.9	35	53	+0.8
Sto	5	59	67	+0.4	35	69	+1.1
WF	5	59	77	+1.0	35	55	+0.9
G540	4	59	70	+0.6	35	44	+0.6
Mc	4	-	-	-	35	40	+0.2
1339	3	59	64	+0.3	35	40	+0.2
Idt	3	-	-	-	35	37	+0.1
C1	2	59	57	-0.1	35	32	-0.2
BM	2	59	81	+1.2	35	66	+1.1
PR	1	59	44	-0.8	35	29	-0.3
MK	1	59	48	-0.6	35	17	-0.9
TR	1	59	37	-1.0	-	-	-
OSF	1	59	46	-0.7	35	21	-0.7
MKxOSF	1	-	-	-	35	9	-1.3
OSFxGB134	1	-	-	-	35	21	-0.8

* Resistance as indicated: 1, highly resistant; 2, resistant; 3, intermediate; 4, susceptible; 5, highly susceptible. (From Wellhausen, 24)

of this section. S15 stock was used as the standard by which selection was judged, that is, the common stock with which all other strains were mixed. Results are presented in table 8. The rated virulence was determined on GB797.

From this experiment it is shown that the direction and rate of change in the S15 type organism when passed through a common host (OSF or GB134) are dependent upon the virulence of the bacterial strain with which it is mixed. The more extreme the virulence of the variant with which S15 is mixed as compared to the virulence of S15, the more rapid the shift in the bacterial population. On passage through the susceptible line selection was in favor of S15 when the variant with which it was mixed was more virulent; against S15 when the second strain was less virulent. In the resistant host, OSF, the converse was true. The experiment was not repeated since the results are consistent and entirely comparable with the more extended S15-A14 mixtures.

These experiments in which host resistance and bacterial virulence were varied show that during host passage the direction and the rate of change of virulent and avirulent bacterial types in a population is dependent upon both host resistance and bacterial virulence. Where either resistance or virulence is held constant such a change is a direct function of the other; where both may vary, as would be true in a

natural population, the change is due to an interaction of both variables.

Bacterial growth in xylem exudate and expressed juice

During passage the living host effects a differential selection of virulent and avirulent bacteria. If either the expressed juice of the entire plant or xylem exudate obtained under root pressure would give a selective reaction upon bacterial growth *in vitro* comparable to the *in vivo* reaction, this differential bacterial growth reaction would be a biological tool useful in studies on the nature of host resistance. For purpose of comparing the *in vitro* and *in vivo* reaction the relative growth of virulent and avirulent strains of *B. stewartii* was ascertained in xylem exudate and expressed juice of certain resistant and susceptible lines of maize.

Xylem exudate was obtained under root pressure from the cut stumps of 3-4 weeks old plants, grown in 6-inch pots, cut off about 2 inches from the base and exudate collected under sterile conditions. Collections were made at 12 and 24 hour periods. All the exudate collected during a period from a given line was bulked into one sample, and this sample divided into as many parts as desired. Sterilization was accomplished by Berkefeld filters and autoclaving at 15 pounds for 15 minutes.

TABLE 8. Change in proportion of S15 type bacteria during 16-day passage through susceptible and resistant host and average daily rate of change.

Stock Mixed With S15	Virulence Index of Pure Culture	Initial Proportion of S15 Bacteria	Susceptible Host-GB134: Proportion of S15	Susceptible Host-GB134: Average Daily Rate of Change	Resistant Host-OSF: Proportion of S15	Resistant Host-OSF: Average Daily Rate of Change
S15	70	-	-	-	-	-
11	20	54	68	+0.9	39	-1.0
A14	24	57	68	+0.7	51	-1.1
93	37	50	59	+0.6	38	-0.8
83	44	45	50	+0.3	40	-0.3
RM	52	62	68	+0.4	57	-0.3
47	63	42	44	+0.1	40	-0.1
16	86	48	45	-0.2	53	+0.3

Expressed juice was obtained from 3 week old plants grown in greenhouse flats or benches. Juice was pressed from plants in a hydraulic press under 12,000 pounds pressure and centrifuged at 4,000 RPM for 10 minutes; the cleared liquid was decanted and sterilized either by autoclaving or filtering through a Berkefeld "N" filter. Plant juices were stored at 2° C after sterilization.

Equal parts of 18-hour old broth cultures of virulent (A14) and avirulent (S15) bacteria were mixed, diluted 1:10,000, and 0.1 c.c. of this dilution introduced into sterile 5 c.c. samples of plant juices. Bacterial counts were made at the time of seeding both as the actual number of bacteria per c.c. of inoculum and the relative proportion of virulent to avirulent organisms in the inoculum. A second count was made again after the desired period of growth, and both the quantitative and relative numbers of bacteria per c.c. of test material determined. The actual number of bacteria was estimated by the poured plate method; the proportion of virulent to avirulent bacteria by the surface smear plate technique. Virulent type A14 and avirulent type S15 bacteria were used for the work.

Data for the actual and relative growth of bacteria in exudate and filtrate from resistant and susceptible maize lines are given in table 9. In general these data show that in

exudate and expressed juice from the resistant host the virulent type grows more rapidly than does the avirulent one.

Since the bacteria that grow in both media are assumed to be identical, the differential growth is apparently due to a nutrient present in one host that is not present in the other. It is believed that the differential growth rate of bacteria in mixed cultures may be used to give some indication of the nature of resistance or susceptibility. Apparently resistance is affected by a thermostable substance soluble and extractable, to some degree at least, in the aqueous plant juice.

Chemical analyses of xylem exudate

The kinds and proportions of host materials in the vascular bundles that may be used for bacterial growth appear important since, at first, the bacteria are apparently limited to the vascular system and also as indicated by the symptoms produced in resistant and susceptible plants. For the first four to seven days after inoculation, the symptoms produced on resistant and susceptible plants are identical, after which time the susceptible plants begin to show signs of complete and rapid death, while resistant plants localize the lesions to the leaves already expanded or expanding, and outgrow the organism. Such symptoms might be expected if amounts of food materials were the limiting factor. Analysis of the vascular

TABLE 9. Relative growth of virulent and avirulent bacteria in xylem exudate and expressed juice.

Material	Treatment	Host Resistance*	Hour's Growth	Proportion of Avirulent Organism: Initial	Final
Test 1. 5/12/38					
Exudate OSF	Filtered	1	19	64	41
Exudate OSF	Filtered + 1% dextrose	1	19	64	53
Exudate OSF	Filtered + 1% peptone	1	19	64	27
Exudate OSF	Filtered + 1% dextrose + 1% peptone	1	19	64	12
Exudate OSF	Autoclaved	1	19	64	47
Exudate GB134	Filtered	5	19	64	69
Exudate GB134	Autoclaved	5	19	64	87
Test 2. 6/4/38					
Exudate OSF	Filtered	1	90	13	0
Exudate OSF	Autoclaved	1	90	13	0
Exudate MK	Filtered	1	90	13	1
Exudate MK	Autoclaved	1	90	13	0
Exudate BM	Filtered	2	90	13	46
Exudate BM	Autoclaved	2	90	13	32
Exudate GB134	Filtered	5	90	13	69
Exudate GB134	Autoclaved	5	90	13	82
Exudate WF	Filtered	5	90	13	70
Exudate WF	Autoclaved	5	90	13	54
Expressed juice OSF	Filtered	1	90	13	2
Expressed juice OSF	Autoclaved	1	90	13	4
Expressed juice GB134	Filtered	5	90	13	44
Expressed juice GB134	Autoclaved	5	90	13	57
Test 3. 9/1/38					
Expressed juice OSF	Filtered	1	60	38	17
Expressed juice OSF	Autoclaved	1	60	38	28
Expressed juice GB134	Filtered	5	60	38	59
Expressed juice GB134	Autoclaved	5	60	38	70

* Wellhausen (24)

exudate of several lines of corn differing in their degree of resistance has been made in an effort to determine if resistance or susceptibility is a matter of food materials in the xylem sap.

Xylem exudate was collected from plants grown in 8" pots to various stages of maturity. These were cut off 3-4" from their base, the pot tipped on its side, and the exudate led into a glass vial through a rubber tube slipped over the cut end of the stalk. No attempt was made to collect the exudate under sterile conditions, but all equipment was sterilized. When exudate sugars were not being determined a drop of toluol was added to each vial. Exudate was collected in two parts - the first consisted of the exudate emerging during the first 12 hours; the second after the 12 (to 24) hour period. Exudate from about 250 plants of 8 lines was obtained. The amount of exudate per plant was small averaging about 1 ml. per plant per 12 hours. Exudate was stored frozen until tested.

Other methods for obtaining exudate than by root pressure were tried, namely, air pressure and suction, but both were unsuccessful. Collection of exudate from field grown plants was also attempted, but here the variability in the amount of exudate obtained per plant was so great that this method was considered unsatisfactory.

Individual plant exudates were analyzed for total reducing sugars by the micro-method developed by Stiles, Peterson and

Fred (21) for bacterial cultures. This method is a modified copper-reduction method in which the amount of reduction is estimated by titration of free iodine liberated. This method was checked against a known sugar solution and also against a ferrocyanide method of sugar estimation. The blanks vary a great deal, and the method is not particularly satisfactory for this reason.

Total nitrogen was estimated by a modified micro-Kjeldahl, reduced iron method. The method is the same as outlined by Loomis and Shull (12), differing only in the quantities of materials used. Two ml. of the exudate were placed in the Kjeldahl, reduced iron powder and 2 drops of conc. H_2SO_4 added. After 14 minutes at room temperature, the flask was warmed for 5 minutes and 18 drops of conc. H_2SO_4 added together with 20-30 mg. of a sodium sulphate-copper sulphate powder mixture. After a short digestion, the ammonia formed was distilled off into 0.005 N. HCl and titrated against 0.005 N. NaOH.

Free ammonia was determined on 10 ml. of exudate by the method given by Loomis and Shull (12), using a micro-Kjeldahl and catching the free ammonia in 0.005 N. HCl. For such a long distillation, clean Pyrex test tubes were filled and left overnight with 0.005 N. HCl, then rinsed with distilled water and the known acid added. This was done to minimize the neutralization of acid by reaction with the glass tubes.

Analyses were made on plants 19 and 42 days old. Usually 5 plants from each line were tested individually for reducing sugars, as well as a sample from the entire mixed collection of exudate for a given line. These were done with exudate from the first and from the second collection. As no appreciable differences were found, an average of all analyses is given in table 10.

For nitrogen analyses, 2 ml. samples were used for total nitrogen and 10 ml. samples for free ammonia. Results of nitrogen determinations are given in table 10.

These data show differences between lines, but such differences are not associated with the degree of resistance or susceptibility of a line. As an inbred line is extremely uniform in its reaction to B. stewartii the individual variation of the plants has been considered of secondary importance and emphasis has been placed on line reaction rather than individual reaction and variability.

Natural rate of mutation in Bacterium stewartii

In preceding sections data have been presented showing that by host passage a change in virulence of bacterial populations is brought about, the direction and rate of this change being dependent upon host resistance. Where the initial inoculum originated from several colonies it has been shown that such cultures are composed of bacterial variants with differing

TABLE 10. Carbohydrate and nitrogen analyses of xylem exudate from several maize lines differing in degree of resistance to Bacterium stewartii.

Line or Cross	Age in Days	Degree of Resistance*	Number Plants Used	Mg. Red. Substances Per Ml. Exudate	Mg. total N Per Ml. of Exudate	Mg. NH_3 N Per Ml. of Exudate
MK	42	1	24	.152		.0182
OSF	42	1	40	.212	.0374	.0208
OSF	19	1	20	.294	.0412	
848	42	2	20	.362		
Idt	42	3	20	.061		
StoxW134	42	3	20	.256		.0100
GB134	42	5	40	.038	.0374	.0093
GB134	19	5	20	.214	.0412	
WF	42	5	24	.383		
Sto	42	5	30	.242		

* Resistance as indicated: 1, highly resistant; 2, resistant; 3, intermediate; 5, very susceptible. (From Wellhausen, 24)

degree of virulence (A88 experiment). For such cases selection explains the known facts of virulence change. When single-cell cultures are used (as was done with A14 stocks) some origin for the variability upon which selection works must be postulated. Since mutation is one known method for emergence of variability, it is important to ascertain if mutations actually occur, their frequency, and the nature of such mutations.

All strains of B. stewartii used for mutation work were purified genetically by repeated single-celling. Agar colonies from such cultures were extremely uniform as to color, size, shape, and surface character. Bacteria from a single colony were used to inoculate 10 c.c. of nutrient broth, a single, different colony being used for each tube of broth. These tubes were incubated at 27° C. for 18-20 hours, shaken repeatedly, diluted and plated. The number of plates poured from each tube of broth varied from 20 to 125. Plates were incubated at room temperature for 3 days and examined under a binocular dissecting microscope at 16 diameters. Each tube was treated as a unit. The total number of colonies was estimated by counting 1/10 of the area of a representative number of plates (i.e., from 10 to 25 per cent of the plates poured from each tube). Each observed variant was put into broth, replated and the variant characters rechecked on this subplate before it was considered that a mutation had occurred. When a variant occurred repeatedly within a tube, only the first 8-10 colonies observed were replated. A culture of all variants from each

tube was kept for future use. Platings from each tube were made again after 30 days, primarily to determine if new types of variation had occurred since the initial 18-hour plating, but also to determine how the observed variants grow under the environment in which they originated, and whether an aging effect could be determined.

From the preliminary work it was apparent that the mutation rates for a given character varied between strains as much as did the mutation rate for different characters within a strain. Because of this strain difference the data for each strain are presented separately and a maximum and minimum mutation rate calculated. For calculation of the maximum mutation rate the total number of colonies under observation was divided by the number of mutant colonies observed. This is considered reasonable since after a mutation arose, that mutant would increase in some ratio to the parent stock, the actual number of mutants observed depending upon the cell generation in which the mutation had originated and whether the mutation had originated in more than one cell. For the minimum mutation rate it has been assumed that a given mutation arose only once in a given tube, and the repeated observations of that mutant merely indicate its later growth. For any interpretation of these data it must be remembered that most variables tend to keep the mutation rate at a minimum. Most important of these are the initial selection of typical colonies

for seeding tubes, the inability of the observer to find all variation present on the agar plate, the errors of random sampling, the possible lethal effects of mutation upon the cell in which it originated, a possible complicated type of segregation of the mutant gene from the heterozygous condition, and a slower growth rate of the mutant than of the normal. If the mutant grew at a faster rate, this would tend to lower the maximum rate somewhat, but not to affect the minimum rate as calculated here, except as growth rate would affect sampling.

Stock S15 and two color variants derived from this stock have been used to determine the mutation rate of characters distinguishable from the normal strain by colony type and color. This stock was chosen because it was characterized by rough, raised, dark yellow, small colonies that remained distinct and individual even when the colonies were crowded on an agar plate. The small colonies of this strain gave many more colonies per plate than any of the larger spreading types. Stock 101 is a pale yellow derivative from S15, appearing to vary from the parent only in color. Stock 105 is a white derivative from S15.

Mutation rates in the three stocks are given in tables 11, 12 and 13. Perhaps the most striking observation is the difference in mutation rates of the different stocks, the rate of the white stock being much lower than the yellow stocks. Within the yellow stocks, usually the same characters were observed to vary, yet each stock yielded a type of variability not

found in the other stock. For example, in stock S15 form R3, and in stock 101 form R4 (fuzzy) was unique for that stock. Color changes were most frequent, followed by the smooth mutation, then a rough colony type common to both stocks (R2), and lastly the unique variability of the individual stocks. The color mutations are interesting in showing that there are different intensities of yellowness and that certain factors* are more stable than others. Thus the mutation dark yellow to pale yellow and the reverse mutation occur about equally often, whereas dark yellow goes to white more readily than does the light yellow. White appears to be extremely stable, the reverse mutation never having been observed. The different "rough" colonies observed show that there are not less than 4 distinct and different types. What is classified as "smooth" probably is really several, different, smooth-type mutations rather than the same mutation in all cases. Because of the relation of smoothness to virulence, these smooth mutations are particularly significant.

The mutations observed are apparently stable from the time of origin. A pure culture of the variant type can be obtained directly from the variant colony, no type of segregation having been observed. Mutations arising as sectored colonies are apparently the same as mutations observed as

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*'Factor' is here used with a wider meaning than 'gene'.

TABLE 11. Natural mutation rate of Bacterium stewartii, strain S15 (dark yellow, rough), after 18 hours of growth in nutrient broth.

Culture Tube Number	Total Colonies Observed in Thousands	Number of Mutant Colonies Observed				
		Pale Yellow	White	R2	R3	Smooth
32	53	3	3	0	0	3
33	63	1	0	0	0	2
34	54	3	0	0	0	1
35	57	3	0	0	0	2
36	56	0	0	0	0	3
37	41	1	0	1	0	0
39	54	2	1	0	0	7
40	55	2	1	3	0	0
41	66	5	0	0	0	6
42	60	3	0	0	0	6
43	66	0	1	0	0	0
44	40	2	0	0	0	4
45	60	0	0	0	0	4
46	63	2	1	0	0	3
47	48	1	1	0	1	0
Total	836	28	8	4	1	41
Number of Tubes in Which Mutation Observed		13	6	2	1	11
Maximum rate per Million Individuals		33.3	9.6	4.8	1.2	49.0
Minimum rate per Million Individuals		15.5	7.2	2.4	1.2	13.2

TABLE 12. Natural mutation rate of Bacterium stewartii, strain 101 (pale yellow, rough) after growth in nutrient broth.

Culture Tube Number	Total Colonies Observed in Thousands	Number of Mutant Colonies Observed After 18 Hours of Growth: Dark Yellow	White	R2	R4	Smooth	Sectored	Variation After 30 Days of Growth (About 5,000 Colonies from Each Tube): Ratio Normal:Variant R2
1	316	33	6	0	0	0	1 dark yel.	1:20
2	83	2	0	0	0	3	0	100% R2 type
3	130	0	0	1	0	1	0	1:11
4	64	2	0	0	0	2	0	100% Normal
5	404	66	0	0	2	1	1 dark yel.	1:25; 1 White R2
6	82	2	1	1	1	6	1 dark yel.	100% R2 type
7	88	1	0	0	0	0	0	1:400; *
8	99	6	269	1	0	2	0	1:400; **
9	35	6	0	0	0	1	1 R2 type	1:3
10	40	7	0	0	0	0	1 dark yel.	1:16
11	16	3	0	0	0	0	0	1:45
12	44	2	2	0	0	0	1 dark yel.	1:12
13	8	2	0	0	0	0	0	1:50*
14	14	3	0	0	0	0	0	1:500
15	16	11	0	0	0	0	0	1:11
16	43	0	0	10	0	1	0	1:75
17	32	4	0	0	0	0	1 dark yel.	1:1
18	48	5	0	0	0	0	1 dark yel.	1:400
19	51	0	0	0	0	0	0	1:4*
20	32	5	3	0	0	1	0	1:7
Total	1645	160	281	13	3	15	7 dark yel. + 1 R2	
No. Tubes in which Mutation Observed		17	5	11	2	9	7 dark yel. + 1 R2	
Maximum rate per Million Individuals		97.3	170.8	7.9	1.8	9.1	4.3 dark yel. + 0.6 R2	
Minimum rate per Million Individuals		10.3	3.0	6.6	1.2	5.5		

* Occasional dark yellow R2

** 100:7 Normal R2 vs Small Colony R2

TABLE 13. Natural mutation rate of *Bacterium stewartii*, strain 105 (white, rough), after growth in nutrient broth.

Culture Tube Number	Total Colonies Observed in Thousands	Mutation Observed After 18 Hour's Growth R2	Variation After 30 Days of Growth (About 10,000 Colonies Observed)
21	64	1	23:50 Normal vs Small Colony Occasional R2 Type
22	42	0	Occasional R3 Type
23	42	0	Colonies normal
24	49	0	1 smooth
25	56	0	All colonies normal
26	92	0	Colonies normal
27	75	0	Occasional R2 type
28	72	0	3 smooth colonies
29	56	0	Colonies normal
30	72	0	500:1 Normal vs Small colony
Total	620	1	
Number of Tubes in Which Mutation Observed		1	
Maximum rate per Million Individuals		1.6	
Minimum rate per Million Individuals		1.6	

entire colonies. Mutation in one factor does not cause a mass mutation, for only one character has been observed to mutate at a time. Thus a colony having a color mutation is not morphologically different from the parent stock. This fact and the occurrence of reverse mutation suggests that the mutations observed are not "loss" type mutations such as would be occasioned by a deficiency. These variants maybe point mutations in the genetic sense. Their mutation rates of one to 25,000 to 800,000 are not different from the known rate of many genes of higher organisms.

The demonstration of mutations in B. stewartii furnishes a source of variability great enough to account for the variation in virulence and colony type observed after host passage. This is particularly important in accounting for changes observed in single-cell cultures during host passage.

Virulence of bacterial variants

Since it was of particular interest to the interpretation of virulence change by host passage, several mutations observed to arise in pedigree cultures were tested for virulence. This was the only physiological test made of these variants. Only a single, unreplicated test was made. The virulence of each variant was compared with that of the type stock from which it arose (table 14).

TABLE 14. Virulence indices of mutants from R1 type stock.

Variant		Virulence
Culture Number	Variant Type	Index
From S15 stock (dark yellow):		
Parental type	R1	71
456	Smooth	52
459	Smooth	57
461	Smooth	47
462	Smooth	64
466	Smooth	87
473	Smooth	53
487	Smooth	44
454	White R1	51
455	White R1	45
401	Pale yellow R1	49
407	Pale yellow R1	69
474	R2	64
From 101 stock (pale yellow):		
Parental type	R1	69
409	Smooth	83
446	Smooth	52
438	R2	75
414	R4	54
From 105 stock (white):		
Parental stock	R1	55
422	R2	66
480	R3	83

Stocks from which these mutations arose were all rough type (like S15). All mutations except those marked smooth are still in the rough phase. The increase in virulence discovered in most of the smooth mutations is important and is to be expected from observations of passage cultures. The exceptions need further study. It seems possible for virulence to be high or low in either the rough or smooth phase.

Growth rates of cultures of B. stewartii

Eight stock cultures of B. stewartii were used to determine if growth rate was correlated with virulence. For the actual growth rate measurements, 1 c.c. of a 1:10,000 dilution of a young culture of bacteria was transferred into 9 c.c. of nutrient broth. The initial number of bacteria introduced and the final number after 14 hours growth was determined. Generation time was calculated by the formula:

$$G.T. = \frac{t \log 2}{\log b - \log a}$$

where G.T. is generation time
t is period of growth in min.
log b is $\log_{10}$ of final number of organisms
and log a is $\log_{10}$ of initial number of organisms.

The number of organisms was estimated by dilution plates.

There appears to be no correlation between generation time and virulence (table 15). Apparently this species has a longer generation time than do many of the animal pathogens, and there is a wide variation in generation time.

TABLE 15. Growth rates and virulence of different cultures of Bacterium stewartii

Stock culture	Generation time in hours	Virulence
A14	1.8	23
A87	2.0	52
D2	2.0	-
FB32	2.2	40
D4	2.2	-
S15	2.3	64
D3	2.9	55
023	3.2	28

Experiments for testing sexual fusion in Bacterium stewartii

If bacteria have sexual fusion then recombination and segregation of characters would be possible. This question is fundamental for interpretation of the mutation and virulence change studies of this paper. Gowen and Lincoln (4) have presented data on a technique which furnishes a certain type of information on this question. They grew in mixed broth cultures pure white and yellow strains of B. stewartii, plated from these mixed cultures at intervals, and replated from single cells to observe possible segregation. If fusion were prevalent, some of the plates from the agar colonies should contain both yellow and white colonies. There was no such evidence from their data. A possible limitation of their work is that only two, repeatedly single-celled yellow cultures were tested against a white culture. If bacteria were of + and - strains the probability of testing unlike strains by

this method is not very high. Environmental conditions may also play an important part in reproductive processes. To correct for these two possible limiting factors and to collect more data by this technique, seven cultures that had been carried in stock without any host passage or single-celling were mixed with the white stock and passed through a resistant and a susceptible host. After 18 to 25 day's growth in the host, isolations were made and the agar colonies plated (table 16).

A few yellow colonies showed segregation for white. Further plating from these yellow colonies did not show any segregation. This would be expected if the original colony started from two bacteria, one white strain, the other of the yellow. There is no indication of sexual fusion that may be determined by this method with any of the stocks used. If such is the case whether a culture is derived from a single cell at the time the culture is taken or whether the culture is made from a group of cells 1 or 2 generations removed from a common cell seems unimportant since variation would be limited to such processes as mutation or somatic segregation.

TABLE 16. Colonies after host passage of mixed cultures of white and yellow bacteria plated to test for sexual fusion of white and yellow cells.

White S15 Variant Mixed With the Following Yellow Strains	Number of Single Colonies Plated				Number of Plates Showing Yellow and White Colonies	
	Susceptible Host		Resistant Host			
	Yellow	White	Yellow	White	Yellow	White
A14	135	22	164	27	2	0
023	218	25	160	15	2	0
A87	100	5	112	5	0	0
S15	157	18	103	12	1	0
FB32	120	10	84	10	4	0
D3	62	0	49	0	0	0
Total	792	80	672	69	9	0

DISCUSSION AND CONCLUSIONS

The opinion prevalent among bacteriologists is that host passage maintains or increases bacterial virulence. Recently Wellhausen (26) showed that the genetic constitution of the host is important in determining whether bacterial virulence is enhanced, maintained or decreased.

This has been verified by passing several stocks of Bacterium stewartii through numerous maize lines. Six stocks of bacteria have been successively passed through inbred resistant or susceptible hosts. The results, with minor exceptions, prove that successive passage through susceptible lines decreases virulence, while passage through resistant lines increases the virulence of bacterial stocks.

The virulence of 13 single-celled virulent stocks was decreased by passage through the susceptible host. Passage of these stocks through the resistant host maintained their virulence. Virulence of single-cell cultures change in a comparable manner to the virulence change of stock cultures during host passage.

Virulent changes in mass cultures may be due entirely to host selection of variation known to be present. Quantitative proof of such differential host selection has been

supplied by inoculating susceptible and resistant hosts with mixtures of virulent and avirulent bacteria of known proportions, and at suitable intervals of time determining the direction and rate of change in the proportion of types. It is concluded that direction and intensity of selection for virulence change is largely dependent upon the resistance or susceptibility of the host.

Host passage of 13 single-cell cultures furnishes critical proof that adaptive variation does arise by some means and is subsequently selected.

The mutation rate for colony morphology and color was determined in nutrient broth on three strains of a bacterial stock. The mutation rate of characters studied was entirely comparable with mutation rates of genes determined experimentally in higher forms. Since evidence for sexual fusion in this species is entirely negative, mutation is probably the chief, perhaps only, source of variation.

Mutation and natural selection within the host have been shown to be two important agencies which bring about observed changes in bacterial virulence during host passage. In this respect the mechanisms for the evolution of bacterial virulence are not greatly different from those in other species.

Considering the large number of individuals and generations possible in a few days of bacterial growth, combined with a mutation rate comparable to that in higher forms, it

is entirely possible that selection processes in the micro-environment of the host are capable of effecting the observed changes in virulence of this pathogen.

Virulent bacteria kill the susceptible host in 10-15 days but only stunt the resistant host; avirulent bacteria stunt the susceptible host but in the resistant host soon become limited to a few early lesions. Successive passage of a bacterial stock through either the resistant or susceptible host effects a change in virulence of the bacteria to a point where that host is stunted noticeably but not killed. The host does not eliminate the parasite by outgrowing it. Once this virulence condition is reached, further host passage does not cause an appreciable change in virulence. Over long periods of time selection within the micro-environment of the host would be toward such a point of equilibrium.

The difference between resistance and susceptibility appears to be of a chemical nature rather than a morphological or local tissue immunity reaction. This seems true since growth of mixed cultures of virulent and avirulent bacteria in expressed juice or xylem exudate of resistant and susceptible plants gives a reaction similar to their growth in the living host. Little more is known about the nature of resistance except that the entity in plant juices is thermostable. The investigation of differential bacterial growth in plant juices

seems very promising as a method to study the nature of resistance. Gross chemical analyses for carbohydrates and nitrogen in the xylem exudate did not show differences between resistant and susceptible lines that can be correlated with resistance.

SUMMARY

1. Six stock cultures of Bacterium stewartii were successively passed through resistant and susceptible inbred maize lines. Passage through the susceptible host decreased virulence, while passage through the resistant host increased virulence.
2. Change in virulence during passage was correlated with changes in colony growth on agar plates. Loss in virulence was always associated with a raised, firm type of colony but not necessarily with a change from the smooth to the rough phase. With an increase in virulence the colonies became more spreading, watery and viscid. The change from rough to smooth and from smooth to rough was observed during passage.
3. Virulence of passage stocks could again by changed by reversing the host through which these stocks were passed.
4. Single-cell colonies from an old stock culture show that it is composed of many variants differing in virulence. Successive passage of this stock culture through susceptible maize decreased the proportion of virulent variants while passage through resistant maize increased the proportion of virulent variants.

5. Virulence of a culture is a direct function of the proportion of virulent and avirulent organisms present in the culture.
6. As in stock cultures, virulent single-cell cultures lose virulence by passage through the susceptible host, while passage through the resistant host maintains high virulence.
7. Mixtures of virulent and avirulent bacteria of known proportions were inoculated into susceptible and resistant hosts, and the change in the proportions of bacteria followed at given intervals of time. There was differential selection for the avirulent type in the susceptible host and for the virulent type in the resistant host. With one exception this was true for 14 lines of maize. Within a given host the rate of change in the bacterial population varies in a linear manner with the proportion of avirulent (or virulent) organisms. Both rate and direction of change are functions of host resistance and bacterial virulence.
8. Virulent bacteria grow better than avirulent bacteria in xylem exudate or expressed juice of resistant plants, whereas avirulent bacteria grow more rapidly in juices of susceptible plants.
9. Chemical analyses of xylem exudate for carbohydrates, total nitrogen and ammonium nitrogen from resistant and susceptible lines did not show any differences that could be correlated with resistance to B. stewartii.

10. After 18 hour's growth in nutrient broth the mutation rate of colony color and colony morphology was determined on 3 strains of a bacterial stock. The mutation rate of these characters is of the same order as the known mutation rate of the genes in higher forms. The calculated mutation rate ranged from one in 20,000 to one in 800,000 individuals.
11. Virulence of the mutations varied greatly from the virulence of the parent stock. Both increases and decreases in virulence were noted.
12. There is no correlation between the rate of bacterial cell division and virulence of 8 stock cultures of B. stewartii.
13. Sexual fusion of white and yellow strains of B. stewartii could not be demonstrated by a critical experiment.

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Plate 1

Comparative virulence of S15 stock and passage strains of Bacterium stewartii on a susceptible line of maize 15 days after inoculation.

A. Passage of S15 stock through susceptible host GB134. Rows from left to right: 1, check; 2, inoculated with stock S15; 3, 4, 5 and 6, respectively, are inoculated with S15 passage strains after 2, 4, 6 and 8 successive host passages through the susceptible host GB134.

B. Passage of S15 stock through resistant host OSF. Rows from left to right: 1, check; 2, inoculated with stock S15; 3, 4, 5 and 6, respectively, are inoculated with S15 passage strains after 2, 4, 6 and 8 successive host passages through the resistant host OSF.

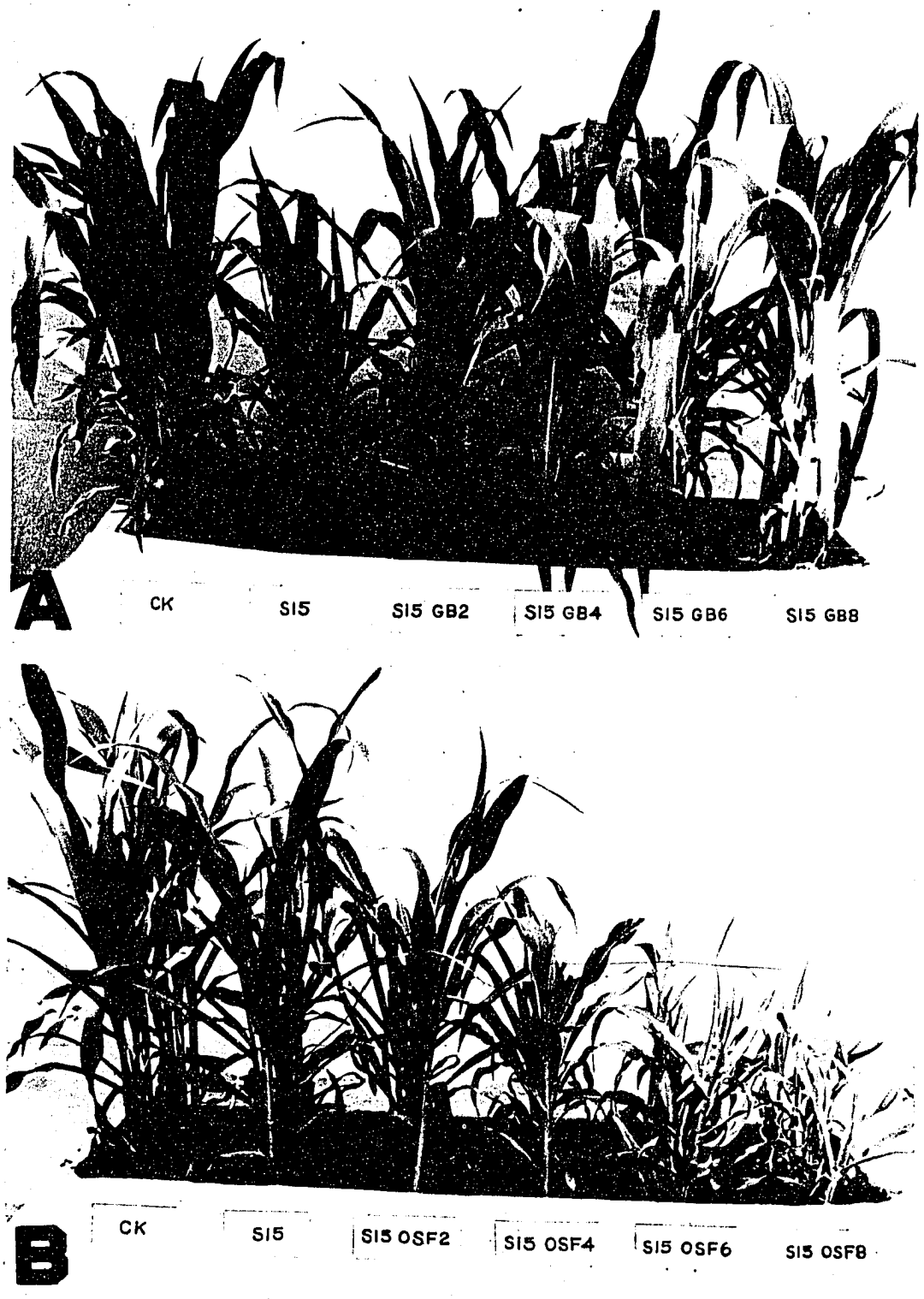
A
CK
SI5
SI5 GB2
SI5 GB4
SI5 GB6
SI5 GB8
B
CK
SI5
SI5 OSF2
SI5 OSF4
SI5 OSF6
SI5 OSF8

Plate 1

Plate 2

Comparative virulence of 023 stock and passage strains of Bacterium stewartii on a susceptible line of maize 15 days after inoculation.

A. Passage of 023 stock through susceptible host GB134. Rows from left to right: 1, check; 2, inoculated with stock 023; 3, 4, 5 and 6, respectively, are inoculated with 023 passage strains after 2, 4, 6 and 8 successive host passages through the susceptible host GB134.

B. Passage of 023 stock through susceptible host WF. Rows from left to right: 1, check; 2, inoculated with stock 023; 3, 4, 5 and 6, respectively, are inoculated with 023 passage strains after 2, 4, 6 and 8 successive host passages through the susceptible host WF.

C. Passage of 023 stock through resistant host OSF. Rows from left to right: 1, check; 2, inoculated with stock 023; 3, 4, 5 and 6, respectively, are inoculated with 023 passage strains after 2, 4, 6 and 8 successive host passages through the resistant host OSF.

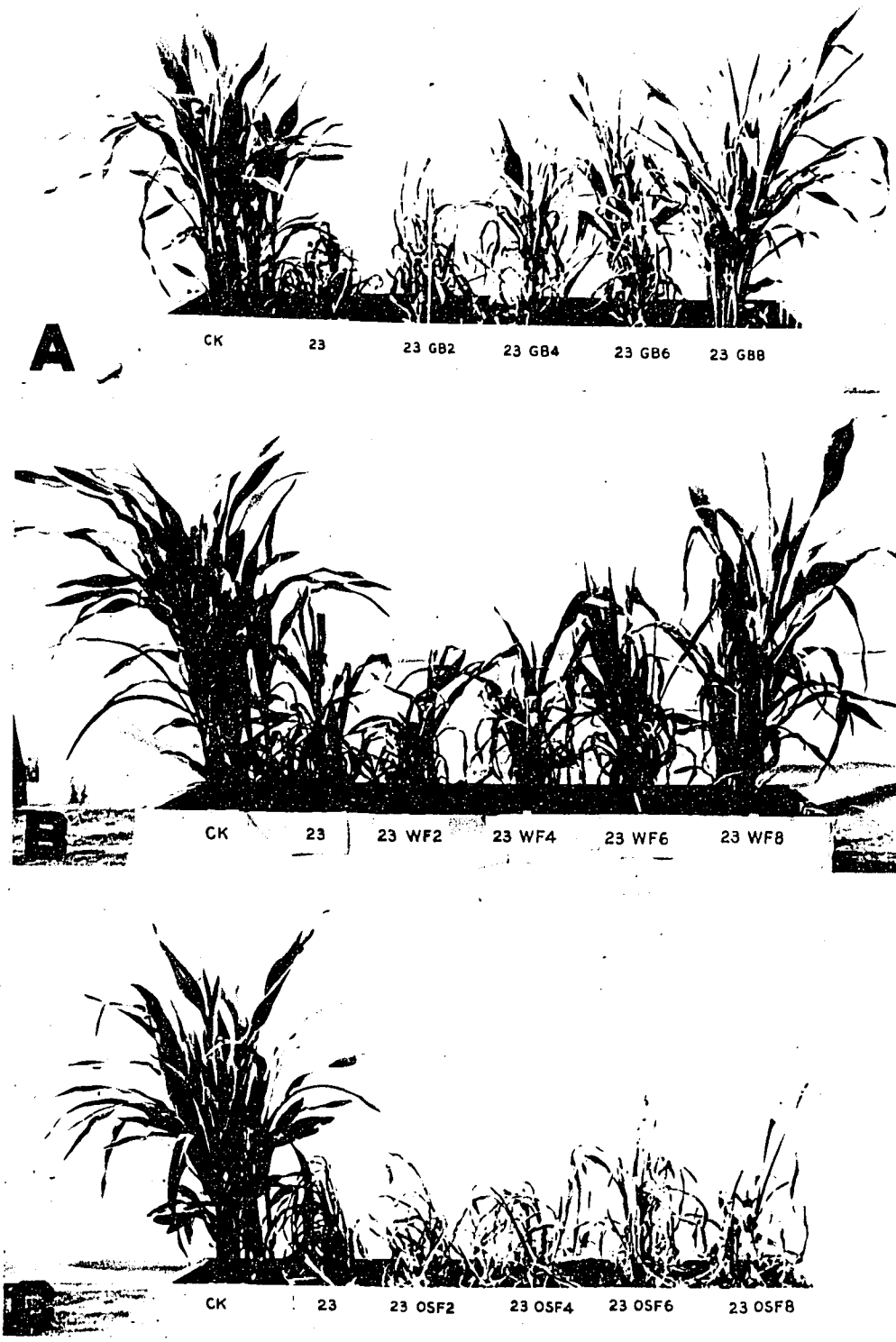
A
CK
23
23 GB2
23 GB4
23 GB6
23 GB8
B
CK
23
23 WF2
23 WF4
23 WF6
23 WF8
C
CK
23
23 OSF2
23 OSF4
23 OSF6
23 OSF8

Plate 2

Plate 3

A. Colony of S15. Note surface roughness.

B. Colony of A14. Note smoothness.

C. Colony of strain 446. A smooth mutant from S15.

D. Colony of strain 453. A smooth mutant from S15.

E. Colony of strain 459. A smooth mutant from S15.

F. Colony of strain 474. R_2 type mutant from S15.

All colonies are 48-hour old surface colonies grown on nutrient dextrose agar. Colony magnifications about 16X.

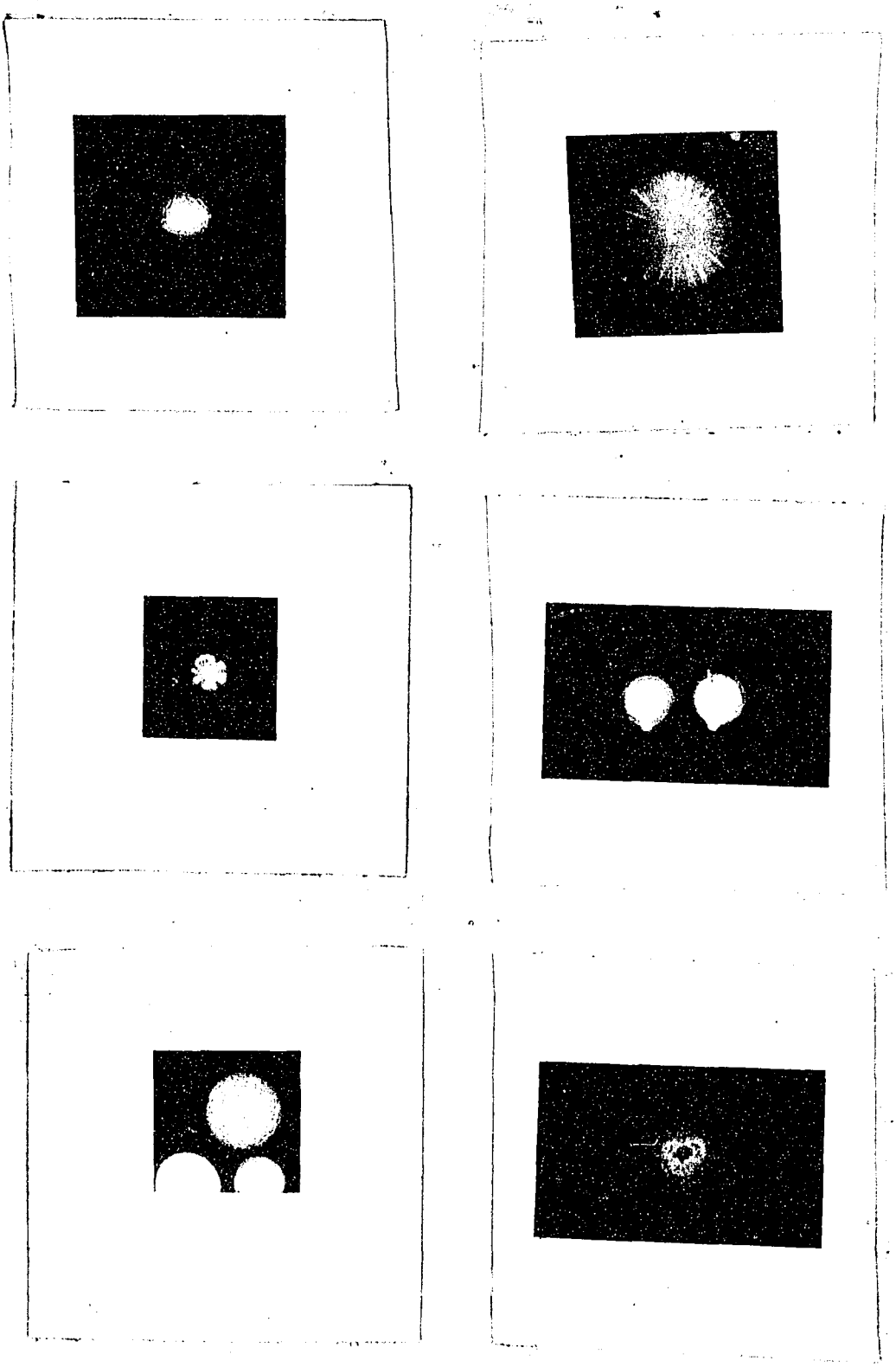

Plate 3